HYDRA Platform Development to Investigate Siglec-engaging Tumor Immunosuppressive Glyco-codes

 # P118

Adam Petrone*, Adam Shoemaker*, Jillian Prendergast, Zakir Siddiquee, Jenny Che, Lihui Xu, Karl Normington, James Broderick, Li Peng

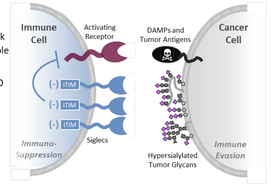
(= authors contributed equally to this work)* *Palleon Pharmaceuticals, Waltham, MA, USA*

Introduction

The glyco-immune checkpoint (Siglec/sialoglycan axis) has emerged as a new mechanism of cancer immune escape and offers new therapeutic interventions to overcome resistance to current immunotherapies. Siglecs (sialic acid-recognizing Ig-superfamily lectins) are type I transmembrane sialoglycan binding proteins expressed on various immune cells (innate and adaptive). Humans express at least fourteen unique Siglecs which have distinct preferred sialoglycan ligands. Tumors upregulate certain sialoglycan patterns to facilitate immune cell evasion by engaging these inhibitory Siglec receptors. This tumor inhibitory "glyco-code" consists of a heterogenous mixture of numerous sialoglycans, binding to Siglecs through low affinity and high avidity interactions. Deciphering the hypersialylation glyco-code of tumors is key to identifying cancer patients for glyco-immune checkpoint blockade therapies. However, the heterogeneity and complexity of sialoglycans make characterization of the tumor surface sialoglycome difficult with current technologies. To overcome this challenge, we developed a proprietary sialoglycan-probing reagent, HYDRA, to functionally detect inhibitory tumor sialoglycans that engage Siglecs. HYDRA mimics this natural avidity-driven Siglec-sialoglycan interaction, consisting of multimeric fusions of a Siglec N-terminal extracellular domain containing the carbohydrate recognition domain (CRD), a trimerization motif, and a Fc dimerization domain. We have generated several HYDRA constructs with robust expression using a mammalian HEK293 system. Size-exclusion chromatography profiles of HYDRA demonstrate high purity and confirmed multimeric assembly. HYDRAs have greater than fifteen-fold increase in binding affinity compared to Siglec-Fc dimers as measured using bio-layer interferometry Octet. HYDRA also demonstrates sialoglycan-specific binding, as its binding was eliminated when cells were treated with sialidase (which removes terminal sialic acids of sialoglycan) or using cells lacking sialoglycans from knocking out UDP-GlcNAc 2-Epimerase. Glycan array binding of HYDRA confirmed similar sialoglycan preferences of its Siglec counterpart as described in the literature, suggesting engineering did not alter glyco-recognition properties. These high-affinity and sialoglycan-specific HYDRAs enabled us to develop a robust immunohistochemistry (IHC) assay to analyze cancer patient samples. A cohort of tissues (>2,500 patients) from various indications were analyzed to enable indication prioritization for glyco-immune checkpoint therapies. HYDRA IHC on healthy and cancerous human tissues demonstrate unique binding patterns with concordance between duplicate primary tumor cores and primary tumor versus metastatic cores from the same patient in non-small cell lung, kidney and colon cancer samples. In summary, the HYDRA technology distills the structural heterogeneity of tumor surface sialoglycans to a straightforward functional readout of immunosuppressive glyco-codes engaging inhibitory Siglecs, which may allow patient stratification based on deciphering a tumor-specific surface glycan pattern.

Glyco-Immune Checkpoints Suppress Innate and Adaptive Immunity

Glyco-Immune Checkpoints in Cancer: *The Siglec/Sialoglycan Axis*



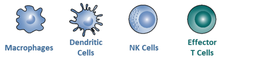
Glyco-Immune Checkpoints:

- Glycan-sensing receptors that block immune activation; play healthy role in self vs non-self discrimination
- Major receptor families: Siglecs (10 unique to human)
- Broad expression across various innate and adaptive immune cells
- Exploited by cancer for immune evasion

Tumor Glycan Evolution:

- Tumors evolve and cloak themselves with functionally immunosuppressive glycan patterns
- Several common tumor glycans: ST, STn, SLe^x, SLe^a, GM2, GD3, PSA
- Recurrent pattern: **Hypersialylation**
- On tumor cell surface glyco-proteins, mucins, and glycolipids

Suppression of Innate AND Adaptive Immunity



→ **Potential to address resistant patient populations**

Tumors are Defined by Glyco-Code

→ **Potential for prospective patient stratification**

Oligosaccharide	R1	R2	R3
N-Glycan	+		
O-Glycan	+		+
Glycolipid	+	+	

◆ = Sialic Acid ● = Gal ■ = GlcNAc ▲ = Fuc

	Relative Recognition by Human Siglec									
	1	2	3	4	5	6	7	8	9	10	11
α2,6 β4 β-R1	-	++	++	-	-	-	-	+	+	-	
α2,3 β4 β-R1	++	-	+	+	++	-	-	+	++	++	+
α2,8 β4 β-R1	+	-	+	-	-	++	+	+	-	++	
α2,3 β3 β-R1	++	-	+	+	-	-	+	+	+	-	
α2,3 β4 α6 β-R1	+	-	+	-	-	-	+	+	-	-	
α2,3 β3 β-R2/α-R3	+	-	+	+	-	-	-	+	-	-	
α2,3 β3 α6 β-R2/α-R3	?	?	?	++	-	-	+	-	+	?	?
α6 β-R2/α-R3	+	+	+	-	-	+	+	+	+	-	

Figure 1: (Top) Schematic representation of glyco-immune checkpoints axis. At the immunological synapse, Siglecs (sialic acid-binding Ig-like lectins) on immune cells interact with tumor- associated sialoglycans and recruit SHP phosphatases, dampening immune responses. Siglecs are expressed on macrophages, monocytes, DC, and NK cells, recognizing a variety of tumor-associated glycans which contain a terminal sialic acid. The Siglec/Sialoglycan plays important roles in cancer cell killing by NK cells and macrophages, cancer antigen presentation, T cell priming and activation, and cancer cell killing by T effector cells. (Bottom) A table adapted from Varki *et al* [1] illustrating the differences amongst several different human Siglec receptors. A "+" symbol denotes detectible binding with "++" being the strong binding and "-" meaning it is very weak or not detectible binding.

Results

Multivalent Siglec Reagents Can be Successfully Purified from HEK Cells

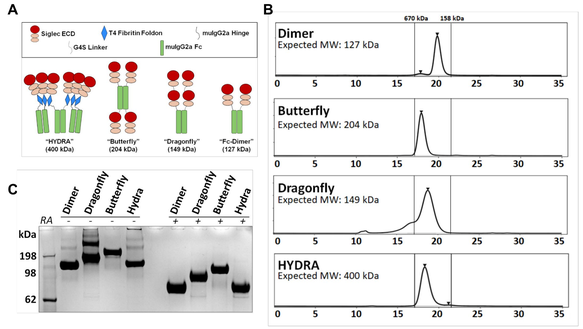


Figure 2: Schematic representations and bioanalytical characterization of Siglec-7 HYDRA, Dragonfly, Butterfly, and Dimer formats. (A) Schematic representation of each of the four formats tested to increase the avidity of the Siglec-7 receptor. (B) Analytical HPLC-SEC traces showing each format successfully purified species running at expected molecular weights. (C) SDS-PAGE gel analysis in the presence or absence of reducing agent, confirming the expected molecular weight.

HYDRA Exhibits Superior Binding Kinetics Using BLI and FACS Analysis

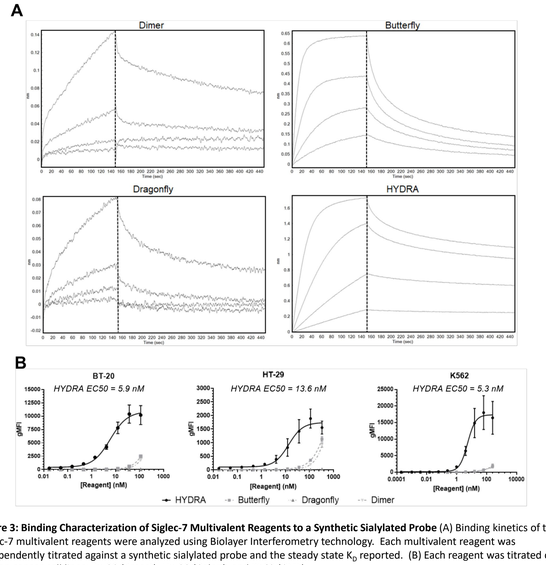


Figure 3: Binding Characterization of Siglec-7 Multivalent Reagents to a Synthetic Sialylated Probe (A) Binding kinetics of the Siglec-7 multivalent reagents were analyzed using Biolayer Interferometry technology. Each multivalent reagent was independently titrated against a synthetic sialylated probe and the steady state K_D reported. (B) Each reagent was titrated onto human cancer cell lines: BT-20 (Breast), HT-29 (Colon), and K562 (CML).

HYDRA is Specific for Sialoglycan Ligands on the Cell Surface

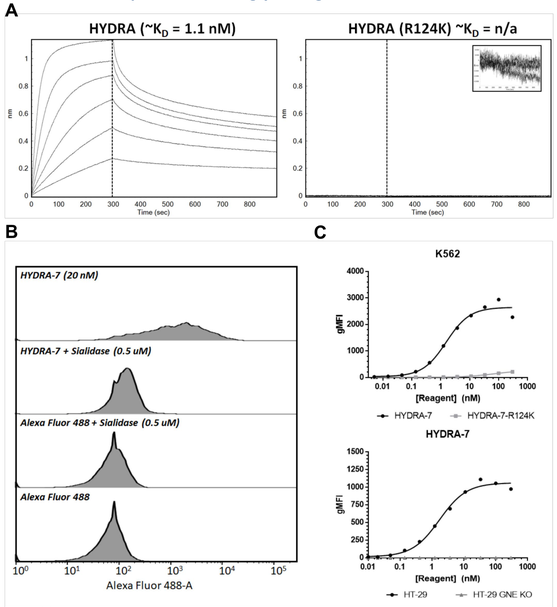


Figure 4: Sialylated Ligand Specificity of the HYDRA Reagent (A) Binding kinetics of the HYDRA reagent and a loss of binding mutant were analyzed using Biolayer Interferometry technology. Each reagent was independently titrated against a synthetic sialylated probe and the steady state K_D reported. (B) HYDRA was used to stain BT-20 cells using FACS in the presence or absence of a sialidase treatment that removes sialic acid from the surface of cells. (C) (Top) HYDRA and the HYDRA-R124 mutant were titrated on K562 (CML) cells. (Bottom) HYDRA was titrated on HT-29 cells as well as a UDP-GlcNAc 2-Epimerase/ManNAc Kinase knockout (GNE KO) line, which lack the ability to synthesize sialic acid.

HYDRAs Retain Siglec Ligand Profiles on Glycan Arrays

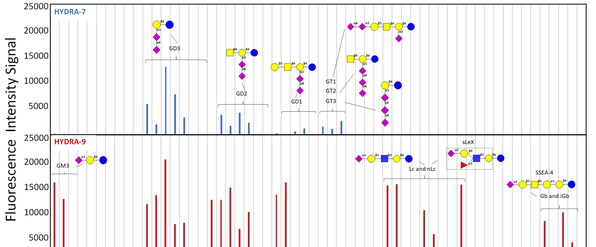


Figure 5: Glycan Arrays demonstrate unique ligand profiles for HYDRAs. 4.8nM of HYDRAs were tested on the commercial Z-Biotech glycosphingolipid array which contains 58 unique epitopes. Fluorescence intensity was measured by GenePix array scanner and background was subtracted using GenePix Pro software. Binding profiles demonstrated differences between HYDRAs.

HYDRA Can be Applied to Decipher the Tumor Glyco-Code in Patients

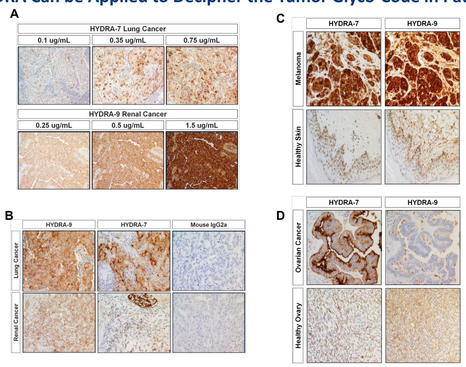


Figure 6: Using HYDRA in an IHC assay can help to decipher the glyco-code of tumors (A) Dilution range and linearity testing for HYDRA-7 and HYDRA-9 on lung and renal cancer tissue. Ideal concentrations were determined to be 0.35ug/mL HYDRA-7 and 0.5ug/mL for HYDRA-9. (B) Accuracy testing for HYDRA-7 and HYDRA-9 as compared to a mulgG2a isotype control on lung and renal tissue. Comparison of HYDRA-7 and HYDRA-9 staining in melanoma (C) and ovarian cancer (d) serial sections as compared to healthy controls.

HYDRA Score Exhibits Primary, Metastatic, and Intra-tumor Concordance

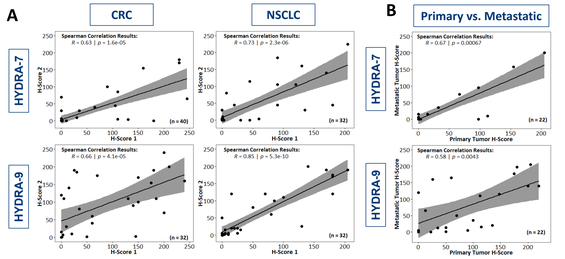


Figure 7: HYDRA IHC concordance. (A) HYDRA-9 and HYDRA-7 concordance from two independent cores in both colorectal cancer (CRC) and lung cancer (NSCLC) tissues. (B) Concordance of HYDRA-9 and HYDRA-7 H-scores between primary and metastatic tissue in CRC, NSCLC, and renal cancer.

Conclusions

- HYDRA platform overcomes low affinity interactions of Siglecs by leveraging high avidity that cannot be achieved by commercial dimer formats.
- HYDRA platform converts the structural heterogeneity of tumor surface sialyloglycans into a functional readout of immunosuppressive glyco-codes.
- Palleon has developed and optimized a HYDRA IHC assay and performed analysis on >1,500 human tumor samples covering over 18 tumor types. Indications are being prioritized for therapies targeting glyco-immune checkpoints.

Acknowledgements

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Cited Literature

1. Varki and Angata. "Siglecs-the major subfamily of I-type lectins." Glycobiology 2006, DOI: 10.1093/glycob/cwj008.